BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau B28

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 15/00, 13/00 — A1

(11) International Publication Number: WO 89/ 02464

(43) International Publication Date: 23 March 1989 (23.03.89)

(21) International Application Number: PCT/GB88/00738

(22) International Filing Date: 7 September 1988 (07.09.88)

(31) Priority Application Number: 8721015

(32) Priority Date: 7 September 1987 (07.09.87)

(33) Priority Country: GB

(71) Applicant *(for all designated States except US)*: AMERSHAM INTERNATIONAL PLC [GB/GB]; Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)* : McKAY, Ian, Alexander [GB/GB]; 17 Sarre Road, London NW2 3SN (GB). MORSE, John [GB/GB]; 25 Woodside Close, Amersham, Bucks. HP6 5EF (GB). McFARTHING, Kevin, Gerard [GB/GB]; 34 School Lane, Weston Turville, Aylesbury (GB). GLOVER, Richard, Peter [GB/GB]; 23 Kerrison Road, Ealing, London W5 5NW (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 5 Quality Court, Chancery Lane, London WC2A 1HZ (GB).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: MODIFYING LIVING CELLS

(57) Abstract

A method of introducing material into living mammalian cells, or of fusing material with the cells, comprises subjecting the cells in liquid suspension in the presence of the material to ultrasonic excitation sufficient to traumatise the cells. The material introduced into the cells, or into a cell membrane, is preferably DNA or RNA or a protein.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		

MODIFYING LIVING CELLS

This invention concerns the use of ultrasound to introduce material into, or fuse material with, living mammalian cells. The technique involves traumatising the cells, but not killing or disintegrating them. Ultrasound involves mechanical vibration at frequencies generally too high for the ear to detect, generally from 18kHz to 20MHz.

Ultrasound has been used for diagnostic and therapeutic purposes on living mammalian tissue. The maximum intensity that can be applied without hazard has been reviewed by W.D.Ulrich (IEEE Transactions on Biomedical Engineering, January 1974, pages 48 to 51).

Ultrasound is widely used to decompose or disintegrate complex chemical species, such as polymers including DNA. Ultrasonic disintegraters for this purpose are commercially available.

Scanning acoustic microscopes use low intensity vibrations in the MHz to GHz range.

A variety of methods of introducing material into living cells exist, including calcium phosphate precipitation and electroporation. Electroporation involves the exposure of cells to a pulsed electric field which presumably creates pores in the plasma membrane. It has been used to introduce DNA into both plant and animal cells, and has been successfully applied to a wide range of cell types which have not been accessible to other methods (G.Chu et al. Nucleic Acids Research, Volume 15 number 3 1987, pages 1311 to 1326).

Similarly, a variety of methods exists for fusing cells, including natural methods involving viruses such as Sendai or HIV, and artificial methods involving

polyethylene glycol mediated fusion or electrofusion. The electrofusion technique comprises two main processes, dielectrophoresis, by which a close contact between cells is established, and electrical membrane breakdown which gives rise to small pores in the cell membranes resulting in fusion of two apposed membranes in close contact. (K.Ohnishi, Journal of Immunological Methods, 100 (1987) 181 to 189.

Ultrasonic forces may be used to achieve the desired close contact between cells. Ultrasound wavelengths may be used that are much smaller than the fusion chamber. This permits not only the production of pearl chains of cells (in a purely propagating wave) but also the concentration of cells at standing-wave pressure maxima. W.M.Arnold et al. (Biochemical Society Transactions, 1986, pages 246 to 249) used 1.0 MHz ultrasound (1mm wavelength) to concentrate erythrocytes or myeloma cells, for fusion by a high voltage pulse.

The present invention provides a method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to the additon of the material, to ultrasonic excitation sufficient to traumatise the cells.

Briefly cells in suspension are exposed to ultrasound frequencies in the KHz to MHz range. These frequencies can induce oscillations in the cells, or cavitation in the vicinity of the cells. The resulting stresses in the cell membranes can allow total disruption of the cell, pore formation in the cell membrane or fusion of two (or more) closely apposed cells. For the duration of this cell disruption, substances in the solution in which the

cells are suspended may be incorporated into those cells.

The cells are subjected to ultrasonic excitation sufficient to traumatise them. This implies that the cells are altered sufficient to effect entry of apposed material and, may be temporarily damaged, but not killed or disintegrated. Probably, pores are momentarily formed in the cell membrane, enabling apposed material to enter the cells or fuse with them. In generating sufficient ultrasound intensity to traumatise the cells, it may be unavoidable that some of them are killed.

The nature of the mammalian cells is not critical. The cells are preferably maintained in suspension in aqueous or other fluid, but may alternatively be treated while adhering to a support.

The ultrasound intensity is chosen to be sufficient to traumatise the cells but without permanently damaging or killing them. Suitable intensities depend on a large number of factors, and are readily determined empirically for a particular experimental set-up. The ultrasound frequency is generally chosen in the range 18kHz to 20MHz. The time of treatment may be chosen empirically, but should not be so long that an unacceptable temperature rise occurs. Treatment may be continuous or in pulses. Total treatment times of a few seconds to a few minutes are likely to be appropriate.

Materials which may be introduced into living cells by this technique include, proteins, nucleic acids, oligonucleotides, DNA, lipids and lipid vesicles. Cells which take up these materials during ultrasonic excitation can survive the process and replicate subsequently. Genes introduced into cells by this technique can be expressed and can be

transmitted to daughter cells in a heritable fashion.

Instead of being introduced into the cell interior, materials may be incorporated by this technique into the cell membrane. Examples of materials for this purpose include lipids, hydrophobic proteins, membrane receptors, lipid vesicles and liposomes. Furthermore, ultrasonic excitation can be used to induce fusion of two or more apposed similar or dissimilar cells to form a single cell.

The cells are preferably maintained in suspension in an aqueous or other liquid medium. Cell concentrations are generally in the range 10^4 to 10^8 cells/ml. Although the material is preferably present at the time of ultrasonic excitation, it may under some circumstances be introduced during or after the excitation while the cells remain traumatised. Preferably, the concentration of the other material should also be kept at a high level. As discussed below, it is possible to use ultrasonic or other techniques to achieve localised high concentration, both of cells and of other material, in a suspension.

Various expedients, known to improve efficiency in the prior art processes of electroporation and electrofusion, are also applicable in the present invention. Adjuvants may be present, including glucose/$CaCl_2$/$MgCl_2$, polyethylene glycol, albumin, calmodulin, phosphatidylserine, glycerylmono-oleate, cholesterol, for cell fusion. The pH, salt concentration and temperature of the suspension are all factors which may affect efficiency. For transfecting DNA into cells, the presence of carrier DNA, such as sonicated salmon sperm DNA, may increase efficiency.

The cells are held in a vessel, whose size and shape and material of construction (acoustic impedance) need to be chosen in relation to the sonicating equipment being used. Small (bijou) polystyrene vials or multi-well tissue culture plates are suitable. Ultrasonic excitation may be provided by one or several ultrasonic transducers, which may be positioned above, beside or below the vessel, or may have a probe which dips into the liquid suspension in the vessel. Or the liquid suspension may be caused to flow in a controlled manner past one or more ultrasonic transducers.

Ultrasonic or other means may be provided for concentrating the cells at particular regions (e.g. standing waves) in the liquid suspension. One ultrasonic transducer may be provided to concentrate the cells, while another is operated periodically to traumatise them. Or the same transducer may be used for both purposes, with the power setting being periodically switched from a low level to a higher one. Control of the ultrasound frequency or frequency spectrum may be used to optimise the fusion of cells or uptake of material.

The following Examples illustrate the invention.

Example 1

Equipment

Soniprep 150 Ultrasonic Disintegrator - (MSE Ltd., Crawley)

Polystyrene bijou vials

9cm tissue culture petri dishes

Cells

63.Ag8.653 mouse myeloma cells suspended in DMEM with 10% calf serum. DMEM is Dubbecco's modified eagles medium.

DNA

pSV2 neo/EcoR1 digest (Southern P., and Berg P., (1982) J. Mol. Appl. Genet., Vol 1, p.327). Mouse carrier DNA: high molcular weight DNA isolated from Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Three amplitude settings were tested for effects on cell viability.

Setting	Approximate % Survial after 10 second pulse
0	100
1	90
3	10

The cells were sonicated at the same settings in three separate bijou (5×10^6 cells in 5ml DMEM/bijou) for 10 seconds in the presence of 20ug pSV2 neo/EcoR1 digest and 50ug mouse carrier DNA.

After sonication, the contents of each bijou were transferred to 6cm petri-dishes and cultured for two days at 37°C on standard tissue culture conditions in an atmosphere of 100% humidity, 90% air and 10% CO_2.

The cells were then transferred to large vessels thus

Setting 0	3 x 9cm petri-dishes
Setting 1	3 x 9cm petri-dishes
Setting 3	1 x 9cm petri-dishes

To each dish fresh DMEM + 10% calf serum was added to make the volume 10ml. The antibiotic G418 was then added to a concentration of 1mg/ml medium. The cells were then incubated for five days before being transferred to larger vessels. Inspection of the petri-dishes before this last transfer revealed

numerous surviving/replicating myeloma cells in the setting 3 dish. Fewer survivors were seen in the setting 1 dish and none were seen in the setting 0 dish. Therefore the pSV2 neo plasmid DNA had entered the sonicated myeloma cells (but not the control cells) and had integrated into those cells in a heritable fashion. This plasmid conferred resistance to the antibiotic G418 on the sonicated cells. The sonicated cells therefore survived in the antibiotic whereas the control cells, which received no plasmid did not.

After a further two weeks the cells still survived in the antibiotic, indicating that the neo gene was integrated into the cells in an heritable fashion.

Example 2

Sonifection of DNA into a Fibroblastic Cell Type

Equipment

Soniprep 150 Ultrasonic Disintegrtor - MSE

Linbro 24 well tissue culture plate - Flow Laboratories

9cm tissue culture petri-dishes - NUNC

Cells

Psi-2 fibroblasts - a retrovirus packaging line (Ref:- Cepko, C.L. et al., Cell 37, 1053-1062, 1984).

DNA

ZNR3 plasmid consisting of a cDNA encoding the human N-ras protein inserted at the Bam H1 site of the pZIP SV(X) plasmid (op. cit.)

Mouse carrier DNA: high molecular weight DNA isolated from Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Psi-2 cells were introduced into the wells of the Linbro plate in suspension at 9×10^5 cells/ml medium (DMEM - Flow Laboratories with 10% donar calf bovine serum - Flow Laboratories).

As in Example 1, the tip of the exponential microprobe of the MSE Soniprep 150 was placed in the centre of the well, in this case at a depth of 1mm below the surface of the medium. Power was applied to the microprobe for 10 seconds in each case. The effects of three different "amplitudes" (as read from the scale on the MSE apparatus) were tested on cell viability:-

Setting	Viable Cells
0	8.7×10^5
1	5.4×10^5
3	1.9×10^5

For the actual experiment two wells were treated at each setting. In each well, in addition to 9×10^5 cells (initially) 10ug ZNR3 DNA and 10ug mouse-carrier DNA were included. After sonication for 10 seconds at each setting 0.2ml of cell suspension was withdrawn from each well for counting. Cell counts were as above (i.e. DNA did not alter cell viability upon sonication in this experiment).

The remaining cells were left to attach to the bottoms of the wells in which they were contained $37^{\circ}C$ in an atmosphere of 100% humidity, 9% CO_2/91% air.

After two days (sonication day 0 - cell splitting day 2) the cells were detached from the bottoms of the wells using a tryspin/EDTA solution (Flow Laboratories 2ml/well).

The cells were transferred to 2 x 10cm tissue culture petri dishes/well, i.e. 4 petri dishes/setting of the sonicator.

9ml fresh medium were added to each petri dish which were then returned to the $37^{\circ}C$ incubator and

incubated as before. The medium in this case, however, contains 1mg/ml G418. Cells which had taken up the plasmid ZNR3 were resistant to the antibiotic as the plasmid contained the same gene as that found in the pSV2 neo-plasmid of Example 1. Medium was changed on day 18. On day 20 the number of colonies of G418 resistant Psi-2 cells on the petri dishes (a colony was considered a group of greater than 16 cells) was counted.

There results were as follows:-

Setting	No. of colonies/4 petri dishes
0	0
1	207
3	0

This gives an efficiency of 10 colonies/10^6 cells surviving sonication/10ug plasmid DNA or $1/10^5$ cells/ug.

Different cell types may need different sonicator settings and different adjuvants to achieve maximum efficiency.

CLAIMS

1. A method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to addition of the material, to ultrasonic excitation sufficient to traumatise the cells.

2. A method as claimed in claim 1, wherein the material introduced into the cells is DNA, or RNA or protein.

3. A method as claimed in claim 2, wherein the material introduced into the cells is a gene, and the cells are subsequently caused to replicate so that the gene is expressed and transmitted to daughter cells in a heritable manner.

4. A method as claimed in claim 1, wherein the material is incorporated into the cell membrane.

5. A method as claimed in claim 1, wherein a mixture of two or more similar or dissimilar cell types is subjected to the ultrasonic excitation to induce fusion of two or more cells.

6. A method as claimed in any preceding claim, wherein the cells are maintained in suspension in a liquid.

7. A method as claimed in claim 6, wherein the cells are concentrated in suspension by means of ultrasonically induced standing waves.

8. A method as claimed in any one of claims 1 to 7, wherein, during and after ultrasonic excitation, the cells are maintained in a suitable form for reintroduction into a host mammal.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00738

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; C 12 N 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 72, 27 April 1970, (Columbus, Ohio, US), A.Sh. Lazaretnik et al.: "Phonophoresis in conditions of experimental tumors and leucoses", see page 187, abstract 88206s, & Fiziol. Zh. (Kiev) 1969, 15(6), 842-6	1
A	Chemical Abstracts, volume 104, 7 July 1986, (Columbus, Ohio, US), V.I. Svidovyi et al.: "Changes in aminotransferase activity and the permeability of erythrocyte membranes in relation to ultrasound and low frequency noise", see page 442, abstract 86056y, & Gig. Sanit. 1985, (10), 73-4	1
A	Chemical Abstracts, volume 104, 7 April 1986, (Columbus, Ohio, US), T.N. Julian: "The effects of ultrasound on drug permeation through ./.	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th November 1988	19 DEC 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/GB 88/00738

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	membrane barriers", see page 393, abstract 116006b, & Diss. Abstr. Int. B 1985 46(2), 532 --	
A	DE, A, 3505161 (GCA CORP.) 21 August 1986 --	
A	Biological Abstracts, volume 78, no. 9, 1984, (Philadelphia, PA., US), V.V. Kuklin et al.: "Using the method of protoplast fusion in the selection of Streptomyces griseus producing grinin, a streptothricin antibiotic", see page 7520, abstract 66698, & Antibiotiki (Mosc) 28(12): 883-889, 1983	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8800738 SA 24184

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/12/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A- 3505161	21-08-86	None	